Molecular Beacons: Probes that Fluoresce upon Hybridization

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We have developed novel nucleic acid probes that recognize and report the presence of specific nucleic acids in homogeneous solutions. These probes undergo a spontaneous fluorogenic conformational change when they hybridize to their targets. Only perfectly complementary targets elicit this response, as hybridization does not occur when the target contains a mismatched nucleotide or a deletion. The probes are particularly suited for monitoring the synthesis of specific nucleic acids in real time. When used in nucleic acid amplification assays, gene detection is homogeneous and sensitive, and can be carried out in a sealed tube. When introduced into living cells, these probes should enable the origin, movement, and fate of specific mRNAs to be traced.

One of the most specific molecular recognition events takes place when a strand of nucleic acid anneals to its complement. A single-stranded oligonucleotide probe can find a complementary strand in the presence of a large excess of other nucleic acids. This process has aided the exploration of gene organization and function and is now being applied to the diagnosis of disease. However, the full potential of this technique has not been realized because measurable changes in the physical properties of nucleic acids that occur upon hybridization are rather small. In order to measure the degree of hybridization, it is necessary to label the oligonucleotide probes, immobilize the hybrids on a solid surface, remove unhybridized probes, and then determine the number of probes that remain. The requirement that unhybridized probes be removed precludes the use of hybridization for real-time monitoring of nucleic acid syntheses and for locating specific nucleic acids in living cells. Furthermore, the need to immobilize hybrids on a solid surface limits sensitivity, since probes bind nonspecifically to surfaces[1]. Several schemes have been put forward for detecting specific nucleic acids in homogeneous solutions[2,3]. Although they are suitable for some applications[4,5], they cannot be used for real-time measurements, nor can they be used in living cells.

Here we report a new principle for the construction of probes that are useful for detecting specific nucleic acids in homogeneous solutions. Probes based on this principle are single-stranded nucleic acid molecules that possess a stem-and-loop structure (Fig. 1). The loop portion of the molecule is a probe sequence that is complementary to a predetermined sequence in a target nucleic acid. The stem is formed by the annealing of two complementary arm sequences that are on either side of the probe sequence. The arm sequences are unrelated to the target sequence. A fluorescent moiety is attached to the end of one arm and a non-fluorescent quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer[6]. The nature of the fluorophore-quencher pair that we prefer to use is such that energy received by the fluorophore is transferred to the quencher and dis-

sipated as heat, rather than being emitted as light. As a result, the fluorophore is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the hybrid formed by the arm sequences. Since nucleic acid double helices are relatively rigid[7], formation of a probe-target hybrid precludes the simultaneous existence of a hybrid formed by the arm sequences. Thus, the probe undergoes a spontaneous conformational change that forces the arm sequences apart and causes the fluorophore and quencher to move away from each other. Since the fluorophore is no longer in close proximity to the quencher, it fluoresces when illuminated by ultraviolet light. We call these probes "molecular beacons" because they emit a fluorescent signal only when hybridized to target molecules. Since unhybridized molecular beacons are dark, it is not necessary to remove them to observe hybridized probes. Consequently, molecular beacons can be used for the detection of specific nucleic acids in homogeneous assays and in living cells.

Results

Design and construction of molecular beacons. We prepared two molecular beacons for the experiments described below. Molecular beacon A contained a 15-nucleotide-long probe sequence and 5-nucleotide-long arm sequences (Fig. 2); and molecular beacon B contained a 35-nucleotide-long probe sequence and 8-nucleotide-long arm sequences. The lengths of the probe sequences were chosen so as to maximize the separation between the fluorophore and the quencher when the probes are hybridized to their targets. This occurs when the probe sequence is 15-, 25-, or 35-nucleotides long, since the open arms of these molecular beacons are arrayed in a *trans* configuration in relation to the probe-target helix.

An ideal fluorophore-quencher pair would be completely unable to fluoresce when the two components are in close proximity. We found that the best available moieties in this regard were the fluorophore 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS) and the quencher 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL). When stimulated by ultraviolet light of peak

Target

+

Molecular
Beacon

↓

Hybrid

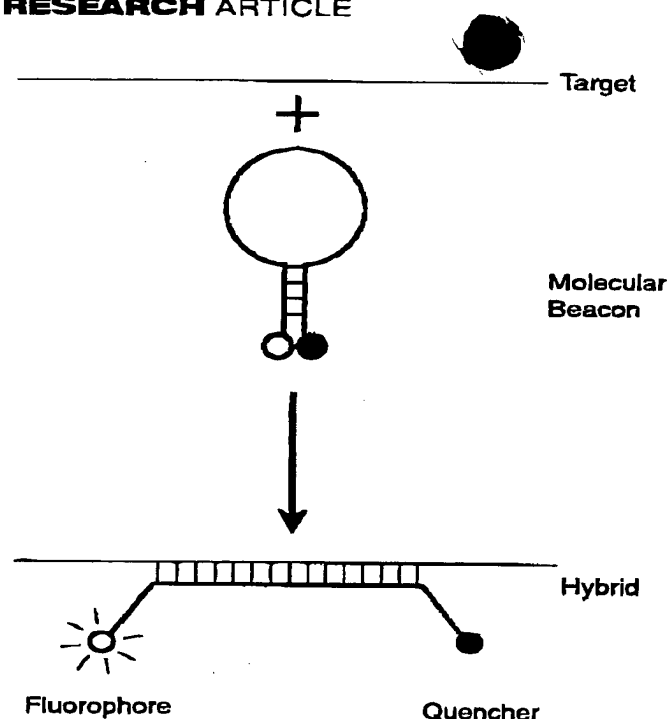Fluorophore   Quencher

EDANS   DABCYL

Figure 1. Principle of operation of molecular beacons. The hairpin stem formed by the complementary arm sequences cannot coexist with the rigid double helix that is formed when the probe hybridizes to its target. Consequently, the molecular beacon undergoes a conformational change that forces the arm sequences apart and causes the fluorophore to move away from the quencher.
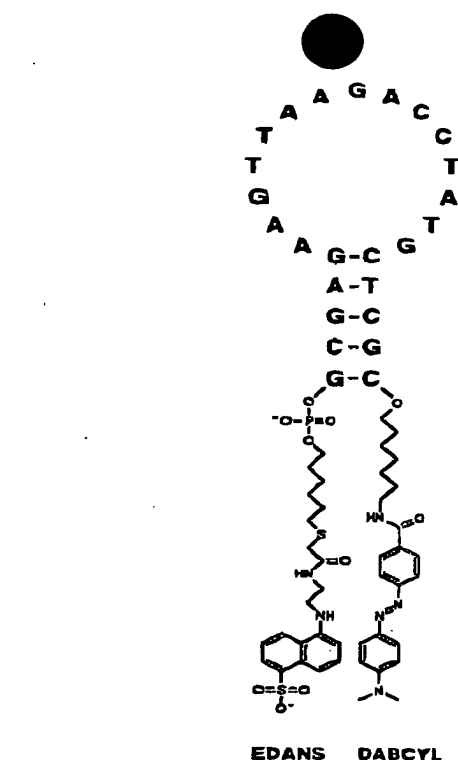
Figure 2. Structure of molecular beacon A. This oligodeoxyribonucleotide (represented by bold letters) consists of a 15-nucleotide-long-probe sequence embedded within two complementary 5-nucleotide-long arm sequences. The fluorophore, EDANS, is joined to the 5'-terminal phosphate by a -(CH₂)₆-S-CH₂-CO- linker, and the quencher, DABCYL, is joined to the 3'-terminal hydroxyl group by a -(CH₂)₇-NH- linker. Although the terminal adducts are schematically depicted on a larger scale than the nucleotide sequence, each adduct is approximately the same size as a single nucleotide.

wavelength 336 nm, EDANS emits a brilliant blue fluorescence of peak wavelength 490 nm, while DABCYL is a nonfluorescent chromophore whose absorption spectrum closely overlaps the emission spectrum of EDANS. In isolation, the radiant energy received by EDANS is stored and then emitted a few nanoseconds later as light of a longer wavelength. However, when EDANS and DABCYL are sufficiently close to one another for resonance to occur, the energy stored in EDANS is efficiently transferred to DABCYL and dissipated as heat. Consequently, molecular beacons that incorporate EDANS and DABCYL are dark when free in solution, yet fluoresce brightly when hybridized to targets. This enables the degree of hybridization in an assay to be monitored with a simple photometer or by visual observation.

As a pair, EDANS and DABCYL exhibit properties that are particularly advantageous for their inclusion in molecular beacons[6]. The mean fluorescent lifetime of EDANS is relatively long (13 nsec), markedly increasing the probability that the EDANS in an unhybridized molecular beacon will transfer its energy to DABCYL before it fluoresces[10]. The distance within which DABCYL is able to quench the fluorescence of EDANS is small compared to the distance that separates them after hybridization to the target has occurred. Furthermore, EDANS is negatively charged and hydrophilic, while DABCYL is neutral and hydrophobic, so they neither attract nor repel each other, and therefore do not alter the intrinsic stability of the stem hybrid. And finally, a sulfhydryl-reac-

tive form of EDANS and an amino reactive form of DABCYL are commercially available and can be covalently linked to synthetic oligodeoxyribonucleotides in simple steps.

We purchased custom oligodeoxyribonucleotides that contained a six-carbon alkyl spacer attached to their 5'-terminal phosphate group and a seven-carbon alkyl spacer attached to their 3'-terminal hydroxyl group. The 5' spacer terminated in a protected sulfhydryl group and the 3' spacer terminated in a primary amino group. We coupled amino-reactive DABCYL to the 3' spacer and sulfhydryl-reactive EDANS to the 5' spacer. The resulting amide and thioester bonds (Fig. 2) were stable under all conditions in which the molecular beacons were used, and the spectral characteristics of EDANS and DABCYL did not change upon coupling to the oligodeoxyribonucleotide.

Conformation of molecular beacons. Molecular beacons exhibit a conformational change upon heating that is consistent with their design. When the temperature of a solution containing molecular beacons is increased, fluorescence increases in a manner that is characteristic of the melting of a nucleic acid double helix. The sigmoidal thermal transition profile (Fig. 3) confirms that at low temperatures the arms form a hairpin stem that brings the fluorophore and quencher together, thus inhibiting fluorescence. However, at higher temperatures, when the helical order of the stem gives way to a random-coil conformation, the fluorophore and quencher tend to be apart from each other, thus enabling the fluorophore to emit a fluorescent signal.

The melting temperature of a molecular beacon depends upon the length of its stem hybrid, the G-C content of the arm sequences, and the concentration of the salts in the solution in which it is dissolved. We found that the melting temperature of a stem hybrid is considerably higher than the melting temperature of an identical hybrid formed from separate molecules. Furthermore, we found that divalent cations, such as magnesium, have a powerful stabilizing influence on the stem hybrid. For example, the melting temperature of a molecular beacon with a 13-basepair stem was 27°C in the absence of magnesium ions, while it was 56°C in the presence of 1 mM $MgCl_2$. Molecular beacons possessing short arm sequences (five to eight nucleotides long) did not form stable stem hybrids in the absence of magnesium ions, yet they formed stable stems in the presence of 1 mM $MgCl_2$. Since assays are almost always carried out in the presence of magnesium, we used molecular beacons containing short arm sequences for our experiments.

Homogeneous detection of hybridization. We found that molecular beacons hybridize spontaneously to their targets at room temperature and undergo a conformational change that results in the generation of fluorescence. When the concentration of both the molecular beacons and the target strands is relatively high, fluorescence develops virtually instantaneously and can be observed with the naked eye (Fig. 4). We studied the development of fluorescence over time with the aid of a spectrofluorometer. An excess of single-stranded oligodeoxyribonucleotide targets was added to a solution containing a relatively low concentration of molecular beacons, and this solution was maintained at a temperature below the melting temperature of the stem hybrid. A 25-fold rise in fluorescence was observed (Fig. 5). This increase exhibited the hallmarks of a classic hybridization reaction: The rise in fluorescence followed second order kinetics and the rate of increase in fluorescence was higher when we increased the molecular beacon concentration, the target concentration, the salt concentration, or the temperature. A similar rise in fluorescence was observed when an RNA target was substituted for a DNA target. When the resulting RNA:DNA hybrids were treated with ribonuclease H (which digests RNA hybridized to DNA), fluorescence returned to the low level characteristic of unhybridized molecular beacons.

The interaction of molecular beacons with their target strands is extraordinarily specific. The addition of nucleic acids that are not complementary to the probe sequence had no effect on the fluorescence of the molecular beacons. Even the presence of a target strand that is perfectly complementary to the probe sequence except for a single nucleotide mismatch in its center did not lead to a rise in fluorescence (Fig. 5). Nor did the addition of a target that contains a single nucleotide deletion in an otherwise perfectly complementary target sequence lead to an increase in fluorescence. Nondenaturing electrophoretic analysis of the products of these reactions showed that hybrids were formed only when the targets were perfectly complementary to the probe sequence.

Real-time monitoring of polymerase chain reactions. When present in sufficient concentration, molecular beacons interact rapidly with target strands. Thus, they can be included in a reaction mixture in which nucleic acids are being synthesized, permitting the progress of the reaction to be followed in real time. In addition to their use under isothermal conditions, molecular beacons may be used to follow the course of reactions that rely upon cyclical increases and decreases in temperature for the synthesis of nucleic acids. Molecular beacons are particularly suited for this purpose because, after they have been denatured at a high temperature, they renature with extremely fast kinetics when returned to a lower temperature. The renaturation of intramolecular hairpins, such as the stem hybrid of a molecular beacon, takes less than a microsecond[11-13], which is substantially shorter than the time it
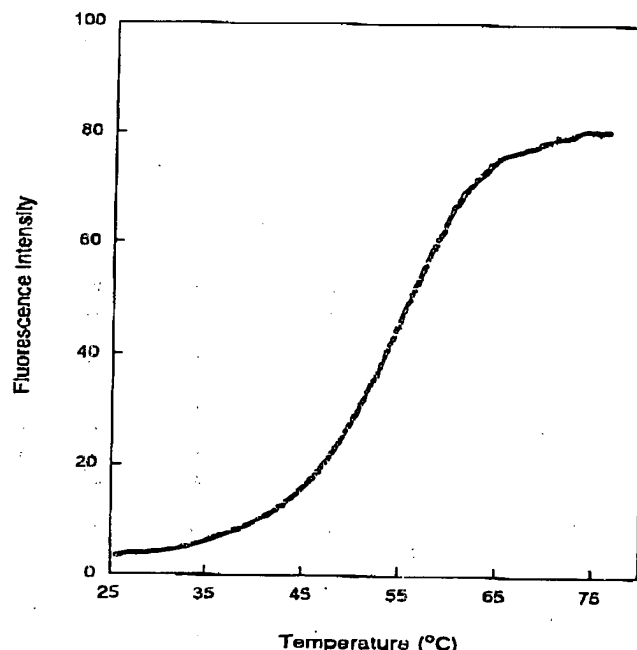

Figure 3. Thermal transition profile of a molecular beacon. The fluorescence intensity of a solution containing molecular beacon A is plotted as a function of temperature on a linear scale from 0 to 100. The minimum intensity was determined from the fluorescence of the buffer alone, and the maximum intensity was determined by adding an excess of target strands to the solution. The increase in fluorescence seen when the temperature was raised is due to a change in the conformation of the molecular beacons from a stem-and-loop structure to a random coil. Because the maximum fluorescence that occurs upon thermal denaturation is lower than the amount of fluorescence that occurs when the probes are fully extended by hybridization to a target, it can be inferred that molecular beacon A is so small that the fluorophore at one end of the random coil occasionally interacts with the quencher at the other end before its stored energy is released as fluorescent light.
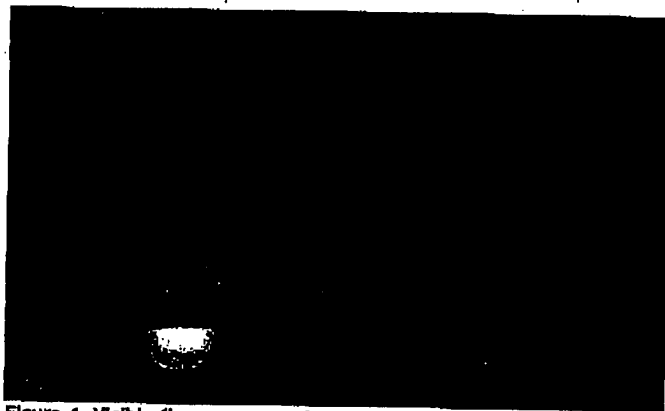

Figure 4. Visible fluorescence of molecular beacons. Two tubes were prepared, each containing 10 µl of a 16 µM solution of molecular beacon A dissolved in 100 mM Tris-HCl (pH 8) containing 1 mM $MgCl_2$. 1.5 µl of a 250 µM solution of a perfectly complementary oligodeoxyribonucleotide target was added to the left tube. Fluorescence was instantaneously visible to the naked eye. The tubes were illuminated with a broad-wavelength ultraviolet light source and were photographed with ASA 200 film for 0.25 sec without using filters.
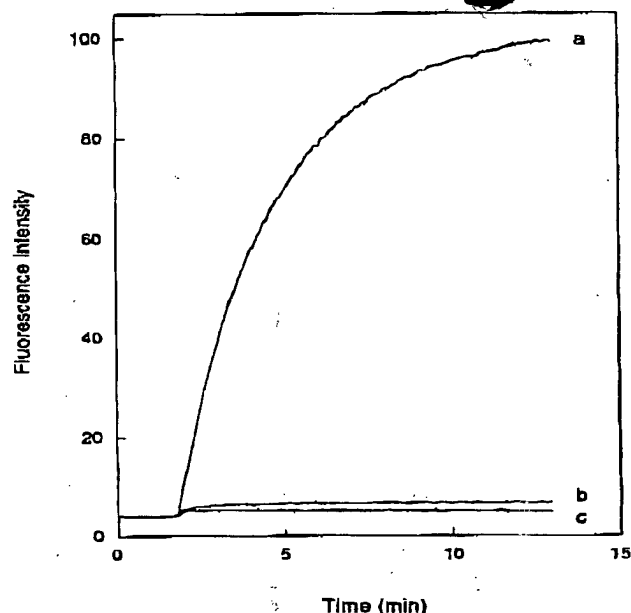
Figure 5. Demonstration of the specificity of molecular beacons. Solutions of molecular beacon A were maintained at 25°C for 2 min. Then, a 5-molar excess of either a perfectly complementary target (trace a), a target containing a single nucleotide mismatch (trace b), or a target containing a single nucleotide deletion (trace c), was added. Upon addition of the perfectly complementary target, the fluorescence of the molecular beacons increased 25-fold, whereas the addition of imperfect targets failed to generate appreciable fluorescence.
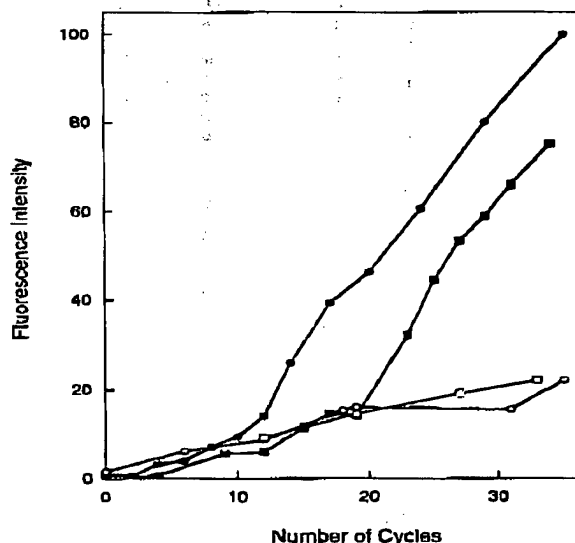

Figure 7. Monitoring polymerase chain reactions in real time. Molecular beacon B was included in four different polymerase chain reactions. One reaction was initiated with 10⁸ template molecules containing a complementary target sequence (●); a second reaction was initiated with 10⁷ molecules of the same template (■); a third reaction was initiated with an unrelated set of template molecules and primers (○); and a fourth reaction was initiated without adding template molecules (□). The reactions were divided into aliquots. Each aliquot was incubated for a predetermined number of amplification cycles, and its fluorescence was measured at 37°C.
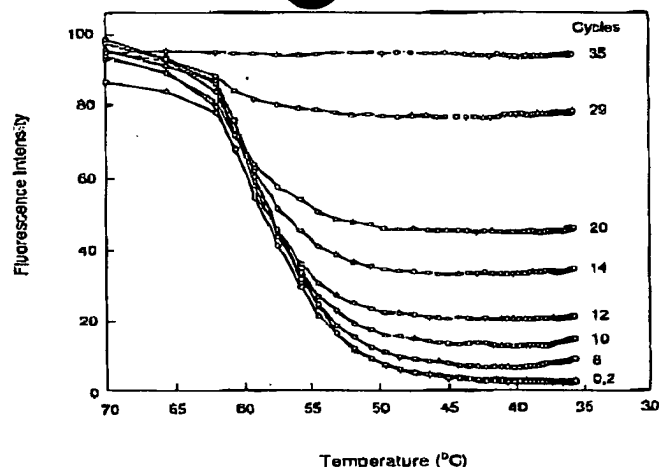

Figure 6. Demonstration that molecular beacons can be used to measure the amount of DNA synthesized in a homogeneous reaction. A polymerase chain reaction was carried out in the presence of molecular beacon B, which was complementary to a sequence in the middle of the DNA being amplified. The reaction was divided into several aliquots. After a predetermined number of amplification cycles, each aliquot was heated to 100°C, placed in a cuvette thermostated at 37°C, and its fluorescence was recorded as the temperature dropped rapidly (65°C to 40°C in 2 min). At high temperatures, the molecular beacons were denatured and they fluoresced brightly. As the temperature dropped, they renatured and became dark, unless there were amplified DNA strands present, in which case they formed fluorescent hybrids. As more cycles of amplification were carried out, the fraction of molecular beacons that remained fluorescent increased, reflecting the increase in the amount of DNA synthesized. A comparison of the fluorescence intensity in each aliquot at 37°C and the amount of DNA synthesized (as determined by polyacrylamide gel electrophoresis and ethidium bromide staining) indicated that there is a strong correlation between these two detection methods and they are about equally sensitive. Furthermore, there was no detectable degradation of the molecular beacons after 35 cycles of amplification.

takes to change the temperature of the solution. Thus, molecular beacons can be present throughout the course of a polymerase chain reaction. The stem hybrids will open when the temperature is higher than their melting point, and they will close rapidly when the temperature is lower than their melting point. When the temperature becomes favorable for hybridization, the molecular beacons will interact with target strands, generating fluorescence. As the target strands synthesized in a reaction accumulate, the fraction of molecular beacons that are bound to targets will increase, causing a brighter fluorescent signal.

We prepared a polymerase chain reaction containing molecular beacons that were complementary to the expected amplification product. Although we did not have a spectrofluorometer in which the temperature of the cuvette could be cycled, we were able to simulate the conditions that would have occurred in a single reaction tube by dividing the reaction mixture into aliquots, subjecting each aliquot to a different number of temperature cycles, and then determining the fluorescence in each. The aliquots were analyzed by heating them to 100°C and then measuring their fluorescence as they cooled. The results (Fig. 6) show that the strength of the fluorescent signal at hybridization temperatures increased as the number of cycles increased. The magnitude of the fluorescence in each aliquot correlated strongly with the amount of amplification prod-

uct that had been synthesized (as determined by subsequent electrophoretic analysis).

Four different polymerase chain reactions, each containing the same molecular beacon, were studied in this manner. The first reaction was initiated with 10^9 template molecules, and the second reaction was initiated with 10^7 template molecules. The third reaction was initiated with a different template and different primers, and the last reaction was initiated without adding template molecules. The results (Fig. 7) illustrate how the fluorescence of molecular beacons can be used in assays designed to measure the original number of template molecules. As expected, fluorescence did not increase above background when unrelated amplification products were synthesized, nor did it increase above background when no templates were present in the reaction. However, in the two reactions in which legitimate target strands were synthesized, the magnitude of the fluorescent signal reflected the number of amplification products that had been synthesized. The fluorescent signal became apparent at about the same time that the polymerase chain reaction entered the linear phase of synthesis. It took an additional seven cycles for the second reaction (which was initiated with 100-fold less template molecules) to enter the linear phase of synthesis than it did for the first reaction. Both reactions followed parallel kinetics. Since the number of cycles it takes for an exponential amplification reaction to enter the linear phase of synthesis is inversely proportional to the logarithm of the initial number of template molecules[14], molecular beacons can be used in assays that determine the initial template concentration over an extremely wide dynamic range. What makes molecular beacons particularly useful is that these measurements can be carried out in real time in a hermetically sealed container, thus reducing the risk of contaminating other reactions. Furthermore, the extraordinary specificity of molecular beacons permits the synthesis of a particular species of nucleic acid to be followed, even when other nucleic acids are synthesized simultaneously.

Discussion

The results confirm that at hybridization temperatures molecular beacons exist in either of two conformational states, depending on whether or not they are hybridized to their targets. Hybridization causes a spontaneous fluorogenic change from one conformational state to the other. The most significant design parameters that affect this conformational change are the length of the arm sequences and the length of the probe sequence. We have observed that in the presence of magnesium ions (which are required for nucleic acid synthesis and function) arm sequences of about 4 to 12 nucleotides form stable stem hybrids, yet they are sufficiently short to dissociate when the probe hybridizes to its target. In addition, we found that the probe sequence should be at least twice the length of each arm sequence to ensure that the conformational change occurs upon hybridization, and to ensure that the fluorophore is sufficiently far from the quencher to restore full fluorescence.

The results demonstrate that there is a 25-fold increase in the fluorescence of molecular beacons upon hybridization to their targets. It should be possible to alter the design of molecular beacons so that even a greater increase in fluorescence can occur, since the fluorescence of EDANS covalently linked to DABCYL is quenched 200-fold[15]. The residual fluorescence of EDANS that we observed in molecular beacons that are not hybridized to their targets could be due to the relatively long alkyl linkers that were used, or it could be due to transient unraveling of the ends of the stem hybrid. Although alkyl linkers are probably necessary for the fluorophore and quencher to find a productive disposition for resonance energy transfer, it may be possible to increase the efficiency of quenching

by shortening the linkers. It may also be worthwhile to link the fluorophore and quencher to nucleotides that are located within the arm sequences, rather than at the end of the arm sequences, to reduce the effects of unraveling.

It is significant that molecular beacons allow the detection of probe-target hybrids without having to separate them from unhybridized probes. This reduces the complexity of assays by enabling the synthesis of nucleic acids to be monitored in real time, in sealed tubes, without performing additional manipulations. Moreover, because molecular beacons are extraordinarily target-specific, ignoring nucleic acid target sequences that differ by as little as a single nucleotide, they are superbly suited for use in assays that identify genetic alleles or particular strains of infectious agents.

The specificity of molecular beacons should enable the detection of many different targets in the same solution. One way to achieve this is by the simultaneous use of several molecular beacons, each of which emits light of a different wavelength, or which can be excited by light of a different wavelength. These molecular beacons can be synthesized using fluorophorequencher pairs other than EDANS and DABCYL. Among suitable pairs are fluorescein and rhodamine, fluorescein and pyrenebutyrate[9], fluorescein and eosine[16], anthranilamide and nitrotyrosine[16], coumarin and ethidium[17], and a number of different terbium chelates and tetramethylrhodamine[18]. Although, the fluorescence of these labels can be distinguished by combining excitation-based and emission-based detection schemes, this approach is suitable only for situations in which the number of targets is limited.

However, there is a way to detect a large number of different targets in a single sample. Because molecular beacons only undergo a conformational change when they fluoresce (rather than having their fluorophore enzymatically cleaved from the probe molecules, as occurs with "taqman" probes[19-21]), many different molecular beacons, each specific for a different target sequence, and each labeled with EDANS and DABCYL, can be covalently linked to different positions on the surface of a reaction vessel or a dipstick. For example, a two-dimensional array of immobilized molecular beacons could be used in a single assay to carry out an extensive survey of an amplified genomic region. Furthermore, arrays of molecular beacons could simultaneously determine the alleles that are present at distant loci. This is important for the diagnosis of genetic diseases and for discriminating closely related pathogenic organisms in clinical samples.

Molecular beacons should also be suitable for the detection of specific nucleic acids within living cells. Although the presence of the fluorophore and quencher should protect molecular beacons against degradation by exonucleases, it may be necessary to modify the nucleotides or the internucleotide bonds to provide protection from endonucleases. Since there is no need to remove unhybridized probes prior to viewing fluorescence, molecular beacons can serve as vital stains, enabling the origin, movement, and fate of specific mRNAs to be traced.

Experimental Protocol

succinimidyl ester of DABCYL (Molecular Probes) dissolved in N,N-dimethyl formamide. The succinimidyl ester of DABCYL was added in aliquots to a continuously stirred reaction mixture at 23°C over a 72 h period. The oligodeoxyribonucleotides were then precipitated by adding 0.3 ml of 3 M sodium acetate, 1.7 ml H₂O, and 7.5 ml ethanol, in order to remove unreacted DABCYL. In the second coupling reaction, EDANS was covalently linked to the 5'-sulfhydryl group of the oligodeoxyribonucleotide previously coupled to DABCYL. Before coupling with EDANS, the S-trityl protective group was removed. The precipitated oligodeoxyribonucleotides were dissolved in 1 ml of 0.1 M triethylammonium acetate (pH 6.5) and incubated with a 5-fold molar excess of silver nitrate (10 µl of a 0.15 M solution) for 30 min at 23°C. A 7-fold molar excess of dithiothreitol (14 µl of a 0.15 M solution) was then added to this mixture. After a 5 min incubation at 23°C, a precipitate was formed, which was removed by centrifugation. The supernatant containing the modified oligodeoxyribonucleotides was added to 1 ml of a1.5 mM solution of 1,5-iodoacetylated EDANS (Molecular Probes) dissolved in 0.2 M sodium bicarbonate, and then incubated at 23°C for 1 h. The reaction mixture was again precipitated with sodium acetate and ethanol. The oligodeoxyribonucleotides, covalently linked to EDANS and DABCYL, were then purified by high-pressure liquid chromatography. The precipitated oligodeoxyribonucleotides were dissolved in 1 ml of 0.1 M triethylammonium acetate (pH 6.5) and fractionated on a C-18 reverse phase column (Apex), utilizing a linear elution gradient of 0 to 75% acetonitrile dissolved in 0.1 M triethylammonium acetate (pH 6.5), and run for 40 min at a flow rate of 1 ml/min. The fraction that absorbed at 260, 336, and 490 nm was isolated. This material had the characteristic fluorescence of EDANS and exhibited the expected thermal denaturation profile. Two molecular beacons were used for most of the experimental work described here. The sequence of molecular beacon A was EDANS-5'-GCGAG-AAGTTAAGACCTATGCTCGC-3'-DABCYL and the sequence of molecular beacon B was EDANS-5'-GCGAGTGCGCCTTAACTGTAGTACTGGTG-AAATTGCTGCCATTGCACTCGC-3'-DABCYL, where the underlined nucleotides constitute the arm sequences and the intervening nucleotides constitute the probe sequence.

Thermal denaturation profile. Fluorescence measurements were performed on an LS-5B spectrofluorometer (Perkin Elmer), using 1 cm path length QS cuvettes (Hellma) whose temperature was controlled by a circulating bath. The molecular beacons were excited at 336 nm, and fluorescence was measured at 490 nm. The fluorescence of a 150 µl solution of 170 nM molecular beacon A dissolved in 100 mM Tris-HCl (pH 8) containing 1 mM MgCl₂ was monitored as the temperature was increased from 25°C to 75°C at a rate of 2°C/min.

Hybridization. One hundred and fifty µl of a 170 nM solution of molecular beacon A dissolved in 100 mM Tris-HCl (pH 8) containing 1 mM MgCl₂ was maintained at 25°C, and its fluorescence was monitored with time. After confirming that there was no change in fluorescence with time, a 5-fold molar excess (5 µl of a 25 µM solution) of a target oligodeoxyribonucleotide (5'-CATAGGTCTTAACTT-3') was added, and the level of fluorescence was recorded every second. The experiment was repeated with imperfect targets that included either a single nucleotide mismatch (5'-CATAGGTGTTAACTT-3'), or a single nucleotide deletion (5'-CATAGGT-TTAACTT-3'), where the identity of the mismatch and the location of the deletion are indicated by an underline and a dash, respectively.

Real-time monitoring of polymerase chain reactions. Molecular beacon B was used for monitoring polymerase chain reactions. The probe sequence was complementary to the middle region of a 130-nucleotide-long DNA fragment produced in a polymerase chain reaction in which the primers were 5'-CTCTTAAAATTAGCAGGAAG-3' and 5'-TGTAGGGAATGCAAATTCC-3' and the template was a plasmid containing a copy of the integrase gene of the HIV-1 viral genome[23]. Four polymerase chain reactions were prepared and divided into a series of 130-µl aliquots. Each aliquot from the first reaction was initiated with 10⁴ template molecules, and each aliquot from the second reaction was initiated with 10³ template molecules. The third reaction was initiated with a different template and different primers, and the fourth reaction contained no added template molecules. All four reactions contained 10 mM Tris-HCl (pH 8), 1.5 mM MgCl₂, 50 mM KCl, 3.6 µM of each primer, 50 units/ml Taq DNA polymerase (Boehringer), and 270 nM molecular beacon B. Each amplification cycle consisted of 92°C for 2 min, 55°C for 3 min, and 72°C for 3 min, and was repeated 35 times. Each aliquot was removed from the thermal cycler after the completion of a predetermined number of cycles. The fluorescence of each aliquot was then measured at 37°C. The fluorescence

of each aliquot from the first reaction was also measured as a function of temperature. Each aliquot was placed in a cuvette and immersed in a boiling water bath for 2 min, and then placed in the spectrofluorometer. The temperature of the cuvette holder was controlled by circulating water from a bath maintained at 37°C. As the contents of the cuvette cooled, fluorescence was measured as a function of temperature.

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